Using the domestic chicken (*Gallus gallus*) as an in vivo model for iron bioavailability[1]

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ABSTRACT Iron fortification of foods and biofortification of staple food crops are strategies that can help to alleviate Fe deficiency. The broiler chicken may be a useful model for initial in vivo screening of Fe bioavailability in foods due to its growth rate, anatomy, size, and low cost. In this study, we assess the broiler as a model for hemoglobin (Hb) maintenance studies and present a unique duodenal loop technique for direct measurement of intestinal Fe absorption. One-week-old chicks were allocated into Fe-deficient versus Fe-adequate treatment groups. For 6 wk, blood Hb, feed consumption, and BW were measured. At wk 7, birds were anesthetized and their duodenal loops were exposed. The loop was isolated and a nonocclusive catheter was inserted into the duodenal vein for blood sampling. A stable isotope solution containing ^{58}Fe (1 mg of Fe in 10 m*M* ascorbic acid) was injected into the loop. Blood samples were collected every 5 min and for 120 min postinjection and analyzed by inductively coupled argon-plasma mass spectrometry for ^{58}Fe concentrations. In the low-Fe group, Hb concentrations, total body Hb Fe, and BW were lower and Hb maintenance efficiency (indicator for dietary Fe availability) was higher than in the high-Fe group ($P < 0.05$). Iron absorption was higher in the Fe-deficient birds ($P < 0.05$). In addition, expression of proteins involved in Fe uptake and transfer [i.e., divalent metal transporter 1 (Fe uptake transporter), ferroportin (involved in Fe transport across the enterocyte), and duodenal cytochrome B reductase (reduces Fe at brush border membrane)] were elevated in the low-Fe group. These results indicate that this model exhibits the appropriate responses to Fe deficiency and has potential to serve as a model for Fe bioavailability. Such a model should be most useful as an intermediate test of in vivo Fe bioavailability observations in preparation for subsequent human studies.

Key words: broiler, duodenum, iron bioavailability, iron absorption, biofortification

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INTRODUCTION

Iron deficiency is the most prevalent nutrient deficiency worldwide (Stoltzfus, 2001). Strategies for reducing its prevalence include distribution of Fe supplements to at-risk groups, food fortification, and diversification of diets. Regardless of the strategies used, there is a need to assess the bioavailability of Fe in many foods and food products. This assessment often requires in vitro screening of foods and food combinations to define factors influencing bioavailability and refine experimental objectives and hypotheses before in vivo testing (Yun et al., 2004; Hu et al., 2006a,b; Ariza-Nieto et al., 2007). Once in vitro screening is done, animal models are usually attempted to refine experimental plans before the more costly and definitive human efficacy trials.

For many in vivo studies using animals, rodents have been the predominant model for Fe bioavailability but appear to have fallen out of favor in recent years due to relatively high efficiency of absorption from foods that have very low availability in humans (Patterson et al., 2008). Piglets have been used as a model but have both strong similarities and differences to human gastrointestinal physiology (Patterson et al., 2008). The most readily apparent macroscopic difference between human and porcine is intestine length. The small intestine of adult pigs is around 15 to 22 m, whereas the large intestine has an average length of 4 to 6 m (Tumbleson and Kalish, 1972; Miller and Ullrey, 1987; Emmans and Kyriazakis, 1999; van Rens and van der Lende, 2002). In contrast, the small intestine of a human adult averages around 5.5 to 7 m, whereas the large intestine is around 1.5 m (Martini et al., 1998; Mochizuki and Makita, 1998; Emmans and Kyriazakis, 1999; Patterson et al., 2008).

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Chickens have a shorter intestinal tract relative to humans (total length is 2.171 m; Sturkie, 2000). The avian digestive system has adaptations designed to facilitate flight. Because birds lack teeth and heavy jaw muscles, food particles are swallowed whole and then reduced in size by the ventriculus and gizzard located within the body cavity (Sturkie, 2000). The small intestine is divided into the duodenum, jejunum, and ileum, although these are not distinguishable based on histology or gross observation. Although there is a distinct duodenal loop, the yolk stalk is often used as a landmark to separate the jejunum and ileum (Sturkie, 2000). The duodenum is the primary Fe absorption site, a feature similar to humans (Sturkie, 2000).

The modern broiler chicken is a fast-growing animal that is sensitive to dietary deficiencies of trace minerals such Fe. As such, it holds potential as a relevant model as a source of tissues for in vitro Fe bioavailability studies, in vivo feeding trials, or both.

The objective of this study was to determine if broilers can be a useful model for assessment of Fe bioavailability from foods. In this paper, we document how Fe status of broilers changes in response to dietary Fe deprivation. This was done via measurement of blood hemoglobin (**Hb**), duodenal Fe uptake, and expression of Fe transport proteins such as divalent transporter 1 (**DMT1**; the Fe uptake transporter), duodenal cytochrome B (**DcytB**; reduces Fe at brush border membrane), and ferroportin (a protein involved in Fe transport across the enterocyte). Furthermore, by documenting the above effects, we explore the feasibility of a duodenal loop preparation of broilers as a means to assess single meal Fe availability and the longer term feeding trial (i.e., 4 to 6 wk) as a means to assess long-term Fe bioavailability of test diets.

MATERIALS AND METHODS

Birds, Diets, and Study Design

Thirty-six Cornish cross fertile broiler eggs were obtained from a commercial hatchery (Moyer's Chicks, Quakertown, PA), from a maternal flock 35 wk in lay. The eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator. Upon hatching (hatchability rate was 90%), 10 chicks per 1 m^2 metal pen were randomly assigned to groups and housed in a total-confinement building. Birds were under indoor ambient temperatures and were provided 16 h of light. Each pen was equipped with an automatic nipple drinker and manual self-feeder. All birds were given ad libitum access to water (Fe content was 0.379 ± 0.012 μg/mL) and diet formulated to meet NRC recommendations during the first week posthatch (NRC, 1994). That was done to see if the hatchlings will build up some Fe stores and will not be Fe depleted at the start of the trial. At the age of 7 d, chicks were allocated into 2 treatment groups on the basis of BW, sex, and blood Hb concentration (2 pens of 5 birds per treatment group): a high-Fe group (Fe+) that was fed an Fe-adequate maize (*Zea mays*)-based diet (141 μg of Fe/g of diet) and low-Fe group (Fe−) that was fed an Fe-deficient maize-based diet (51 μg of Fe/g of diet) (Fe as ferric citrate, Sigma, St. Louis, MO). Diet composition is shown in Table 1 (diets were pelleted and fed as crumbles).

Feed intakes were measured daily. Blood samples were collected from the wing vein (n = 10, ~100 mL) using microhematocrit heparinized capillary tubes (Fisher, Pittsburgh, PA). Samples were collected in the morning after an 8-h overnight fast. The samples were analyzed for Hb concentration (see below). Body weights and Hb concentrations were measured weekly.

Iron bioavailability was calculated as Hb maintenance efficiency (**HME**) (Tan et al., 2008; Tako et al., 2009):

$$\text{HME} = \frac{\text{Hb Fe (final)} - \text{Hb Fe (initial)}}{\text{Total Fe intake, mg}} \times 100,$$

where Hb Fe = total body Hb Fe. The Hb Fe was calculated from Hb concentrations and estimates of blood volume based on BW (a blood volume of 85 mL per kg of BW is assumed) (Sturkie, 2000):

$$\text{Hb Fe (mg)} = \text{BW (kg)} \times 0.085 \text{ L of blood/kg} \times \text{Hb (g/L of blood)} \times 3.35 \text{ mg of Fe/g of Hb.}$$

Iron intakes were calculated from feed intake data and Fe concentrations in the feed.

Table 1. Composition of the experimental diets

Item	Fe-adequate diet	Fe-deficient diet
Ingredient, (g/kg)		
Corn	499.5	500
Soybean meal	350	350
Corn oil	30	30
Corn starch	47	47
Vitamin-mineral premix (no Fe)[1]	70	70
Choline chloride	0.75	0.75
DL-Methionine	2.5	2.5
Ferric citrate	0.5	—
Total (g)	1,000	1,000
Determined content		
Fe[2] (μg of Fe/g)	141 ± 5.2[a]	51 ± 1.9[b]

[a,b]Within a row, means without a common superscript are significantly different ($P < 0.05$).

[1]Vitamin-mineral premix provided (per kg of diet): retinyl palmitate, 1,208 μg; ergocalciferol, 5.5 μg; DL-α-tocopheryl acetate, 10.72 mg; menadione, 0.5 mg; D-biotin, 0.05 mg; choline chloride, 0.5 g; folic acid, 0.3 mg; niacin, 15 mg; Ca-D pantothenate, 10 mg; riboflavin, 3.5 mg; thiamin, 1 mg; pyridoxine, 1.5 mg; cyanocobalamin, 17.5 μg; $CuSO_4 \cdot 5H_2O$, 6 mg; $C_2H_8N_2 \cdot 2HI$ (ethylene diamine dihydriodide), 0.14 mg; MnO, 4 mg; Na_2SeO_3, 0.3 mg; and ZnO, 100 mg.

[2]Iron concentrations in the diets were determined by an inductively coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA) after wet-ashing.

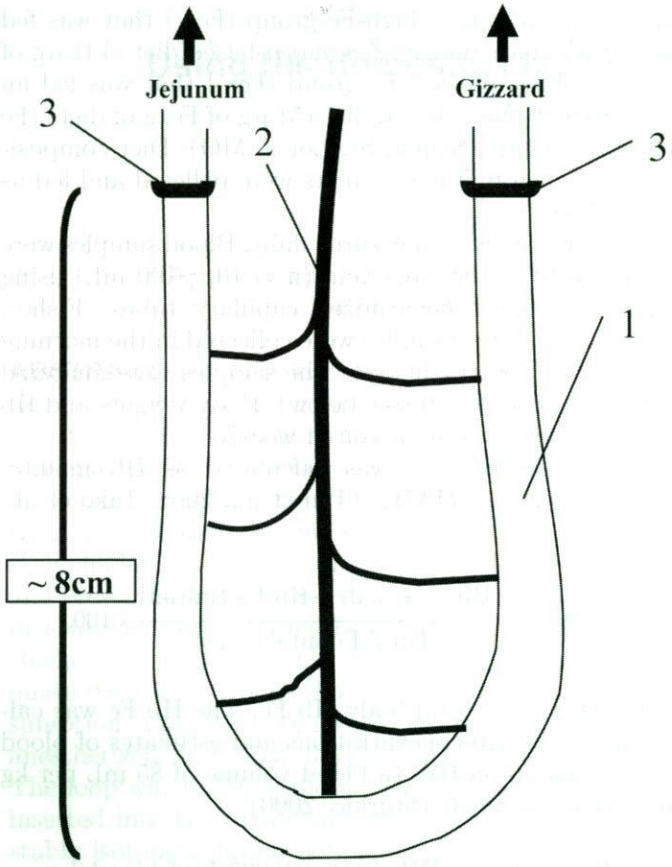

Figure 1. Illustration of the chicken duodenal loop model (frontal view): 1 = stable isotope (^{58}Fe) solution injection site; 2 = duodenal vein (blood collection site); 3 = ligation sites.

Hb Measurements

Blood Hb concentrations were determined spectrophotometrically using the cyanmethemoglobin method (H7506-STD, Pointe Scientific Inc., Canton, MI). The instructions of the kit manufacturer were followed.

Ligated Loop Procedure

At wk 7, birds (n = 3) were fasted overnight and anesthetized by i.m. injection of Dial/Ketamine (a complex anesthetic, 1 mL/kg of BW; Sigma). A small incision in the lower abdomen was made to expose the duodenal loop. The duodenal loop was ligated using a surgical thread (Roboz Surgical, Gaithersburg, MD) at both ends and a nonocclusive 22-gauge catheter was inserted into the duodenal vein. The wing vein was also exposed and a BPE-T50 polyethylene tubing (Solomon Scientific, San Antonio, TX) was inserted into the vein for heparin administration to prevent coagulation in the duodenal cannula during the experiment. Initial blood samples were taken from the duodenal vein. Then each duodenal loop was injected with a 10 m*M* ascorbic acid solution with 1mg of ^{58}Fe (total volume of 3 mL). The anesthetized birds were kept under lamps to maintain their body temperature and wetted gauze pads with warm saline were placed over the loops to maintain their moisture. Blood samples were collected before the stable isotope injection and then every 5 min and for 2 h post solution injection using a pump (Alitea VS-10R, Precision Instrumentation Ltd., Englewood, CO) that was set to draw blood at a rate of 0.13 mL/min (total blood volume collected was 15.6 mL/2 h per bird) using 6-mL Vacutainer heparin-coated tubes (Figure 1). Samples were analyzed by inductively coupled argon-plasma mass spectrometry for ^{58}Fe concentrations. At the end of procedure, an overdose of the Dial complex was used to kill the birds. Sections (5 cm) of the mid duodenum were immediately taken and scrapings of the duodenal mucosa were isolated and stored in an −80°C freezer until gene expression analysis. All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

Preparation of Fe Isotope Solutions for i.v. Infusion

Enriched stable isotope of Fe (^{58}Fe 92.2%, enriched; Isoflex USA, San Francisco, CA) was used for this study. The ^{58}Fe (in the form of elemental Fe powder) was dissolved in concentrated HCl (Fisher; 200 μL of HCl/mg of Fe). The solution was diluted with deionized water to a final concentration of 1 mg of ^{58}Fe/mL (Tako et al., 2009).

For each bird, a volume of 1 mL of the concentrated ^{58}Fe solution was transferred into a separate vial with 2 mL of the 10 m*M* ascorbic acid (pH 4) solution. The stable isotope solutions were sterilized by filtration and aliquots were stored in sealed, sterile vials until use.

Measurement of Fe Absorption

Iron absorption was estimated from the concentrations of the stable isotope tracer (^{58}Fe) in whole blood relative to ^{56}Fe natural abundance concentration. Blood samples (0.05 mL) were wet-digested in concentrated HNO_3 followed by a 50:50 mixture of HNO_3 + $HClO_4$ and brought to near dryness in a heating block. The ash was dissolved in 15 mL of 2% (0.316 mol/L) nitric acid, and isotope ratios were determined via inductively coupled argon-plasma mass spectrometry (Agilent 7500 CS, Santa Clara, CA; Patterson et al., 2008; Tako et al., 2009). The amounts of Fe-stable isotope (^{58}Fe) in excess of the naturally occurring amounts in the total circulating Hb of the birds were calculated from mass spectrometer isotope ratio analyses of whole blood, Hb concentration measurements, and estimates of blood volume (Frenkel et al., 1972).

Isolation of Total RNA

Total RNA was extracted from 30 mg of the distal duodenal tissue using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc.,Valencia, CA) according to the protocol of the manufacturer. Briefly, tissues were disrupted and homogenized with a rotor-stator homogeniz-

er in buffer RLT (Qiagen Inc.; for lysis of cells-tissues before RNA isolation), containing β-mercaptoethanol. The tissue lysate was centrifuged for 3 min at 8,000 × *g* in a microcentrifuge. An aliquot of the supernatant was transferred to another tube, combined with 1 volume of 70% ethanol, and mixed immediately. Each sample (700 μL) was applied to an RNeasy minicolumn, centrifuged for 15 s at 8,000 × *g*, and the flow-through material was discarded. Next, the RNeasy columns were transferred to new 2-mL collection tubes, and 500 μL of buffer RPE (Qiagen Inc.) was pipetted onto the RNeasy column followed by centrifugation for 15 s at 8,000 × *g*. An additional 500 μL of buffer RPE was pipetted onto the RNeasy column and centrifuged for 2 min at 8,000 × *g*. Total RNA was eluted in 50 μL of RNase-free water. All steps were carried out under RNase-free conditions. Ribonucleic acid was quantified by absorbance at $A_{260/280}$. Integrity of the 28S and 18S ribosomal RNA was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. Deoxyribonucleic acid contamination was removed using Turbo DNase treatment and removal kit following the instructions of the manufacturer (AM2239, Ambion, Austin, TX).

Isolation of Chicken DcytB Gene Fragment

Primers were designed to correspond to nucleotides 496 to 520 (5′-GGC CGT GTT TGA GAA CCA CAA TGT T-3′) and 686 to 710 (5′-CGT TTG CAA TCA CGT TTC CAA AGA T-3′) of the previously published human small intestinal DcytB sequence (GenBank database; GI 20380692). Total RNA was amplified using the Promega Access RT-PCR System (Promega, Madison, WI). The program was as follows: 2 min at 94°C, 1 min at 48°C, 2 min at 68°C for 40 cycles, followed by 7 min at 68°C. The reverse transcription-PCR (**RT-PCR**) products were separated on a 1.5% agarose gel, visualized by staining with ethidium bromide, excised from the gel, and purified using a High Pure PCR Product Purification Kit following the instructions of the manufacturer (20021, Qiagen Inc.). The chicken duodenal DcytB cDNA fragment was subjected to automate sequencing using an Applied Biosystems 373A DNA sequencer (Applied Biosystems Inc., Foster City, CA). Nucleic acid sequences were analyzed and homology between chicken and other DcytB sequences was calculated using DNAMAN version 4 software (Lynnon Biosoft, Pointe Claire, Canada).

DMT1, DcytB, and Ferroportin Gene Expression Analysis

As described previously (Tako et al., 2005), first-strand cDNA were synthesized from 5 μg of total RNA from each bird using oligo $(dT)_{18}$ as primers in the presence of Moloney murine leukemia virus reverse transcriptase (Fermentas Inc., Glen Burnie, MD) for 1 h at 42°C. Polymerase chain reaction was carried out with primers chosen from the fragment of the chicken duodenal DMT1 gene (GI 206597489; forward: 5′-AGC CGT TCA CCA CTT ATT TCG-3′; reverse: 5′-GGT CCA AAT AGG CGA TGC TC-3′), DcytB gene (GI 219943161; forward: 5′-GGC CGT GTT TGA GAA CCA CAA TGT T-3′; reverse: 5′-CGT TTG CAA TCA CGT TTC CAA AGA T-3′), and ferroportin gene (GI 61098365; forward: 5′-GAT GCA TTC TGA ACA ACC AAG GA-3′; reverse: 5′-GGA GAC TGG GTG GAC AAG AAC TC-3′). Ribosomal 18S was used to normalize the results, with primers from the *Gallus gallus* 18S ribosomal RNA (GI 7262899; forward: 5′-CGA TGC TCT TAA CTG AGT-3′; reverse: 5′- CAG CTT TGC AAC CAT ACT C-3′). Determination of the linear phase of the PCR amplification was performed with Tfi-DNA polymerase (Access RT-PCR System, Promega) with pooled aliquots removed at 15, 20, 25, 30, 35, 40, 45, 50, and 55 cycles. Amplification of the chicken duodenal DMT1, DcytB, and ferroportin genes was performed for 32, 33, and 30 cycles, respectively, which consisted of denaturation (95°C, 30 s), annealing (48°C, 1 min), and extension (72°C, 1 min); ribosomal 18S was amplified at 32 cycles under identical conditions in a different tube. Ribosomal 18S (426 bp) and chicken duodenal DMT1-ferroportin PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using a Gel-Pro Analyzer Version 3.0 (Media Cybernetics, Bethesda, MD).

In Vitro Fe Bioavailability Assessment

An in vitro digestion-Caco-2 cell culture model (Glahn et al., 1998) was used to assess Fe bioavailability. With this method, foods or meals are subjected to simulated gastric and intestinal digestion. Briefly, samples (approximately 20 g) were ground to a fine powder by using a coffee grinder. Aliquots of the powders (approximately 1.0 g) were mixed on a rocking platform (~80 rpm) in 15 mL of buffer containing 140 m*M* NaCl and 5 m*M* KCl (pH 2). The pH of the mixture was then adjusted to 2 with 1.0 *M* HCl. Pepsin (0.5 mL of a 20 mg/mL, pH 2 pepsin solution; Sigma P6887, 3,200 to 4,500 units/mg of protein) was added, and the mixture was incubated for 1 h at 37°C on a rocking platform to simulate gastric digestion. The resulting pepsin digest was then adjusted to pH 7 with 0.1 *M* $NaHCO_3$, and a pancreatin-bile mixture [2.5 mL; 0.3 g of pancreatin (Sigma P3292) and 1.8 g of bile extract in 150 mL of 0.1 *M* $NaHCO_3$] was added. The mixture was incubated at 37°C for 2 additional hours on a rocking platform to simulate intestinal digestion. This pancreatin-bile digestion took place in a small upper chamber positioned over a monolayer of cultured Caco-2 cells.

Caco-2 cells (at passage 28 to 35) had been seeded at a density of 50,000 cells/cm^2 and were grown in Dulbecco's modified eagle medium (Gibco, Invitrogen, Carlsbad, CA) at 5% CO_2/95% air, RH, and 37°C. The experiment was conducted 14 d postseeding. Before the uptake assay, cultures were washed with tempered

(37°C) minimum essential medium at pH 7, and 1 mL of minimum essential medium was placed in each well. The contents of the upper chamber were separated from the Caco-2 cell layer by a 15,000 molecular weight cutoff dialysis membrane (Spectra/Por 2.1, Spectrum Medical, Gardena, CA) that allowed Fe released from the digested samples to diffuse into the medium bathing the cells in the lower chamber. After the simulated intestinal digestion, the upper chamber was removed, and the cells were incubated at 37°C for 23 h to allow ferritin to form. The growth medium was removed, and the cell monolayer was harvested from the bottom chamber by adding deionized water and placing it in a sonicator to disrupt the cells. Caco-2 cells synthesize ferritin in response to increases in intracellular Fe concentration. Therefore, ferritin concentration in the cells, measured in an aliquot of the cell suspension by using an enzyme-linked immunoassay (Ramco, Houston, TX), was used as an index of Fe uptake. Cell ferritin was expressed as nanograms of ferritin per milligram of cell protein.

Statistical Analysis

One-tailed Student's *t*-tests were performed to compare differences between means using the JMP software (SAS Institute, Cary, NC). Values were considered significantly different at $P < 0.05$. Values in the text are means ± SEM.

RESULTS

Growth Rates, Hb, and HME

The broilers grew well over the 7-wk study. There were no differences in feed intakes at any time throughout the study; however, Fe intakes and BW were significantly higher in the high-Fe group than the low-Fe group (Table 2). Blood Hb concentrations were significantly higher in the high-Fe group compared with the low-Fe group after the first week of the study (Table 2). The HME values were significantly different between treatments (Table 2). In addition, the increase in total body Hb Fe from the beginning of the study to the end of the seventh week was significantly greater in the high-Fe group (85 ± 7 mg) than the low-Fe group (54 ± 6 mg, $P < 0.05$, data not shown).

Isolation and Sequencing of Partial Chicken Small Intestinal DcytB cDNA

A 118-bp fragment of the intestinal DcytB gene was isolated by RT-PCR and was sequenced. The cDNA sequence of the chicken intestinal DcytB was entered into the EMBL Nucleotide Sequence Database (http://www.ncbi.nlm.nih.gov/) under accession number GI 188536035. The predicted amino acid sequence of DcytB resulted in a predicted translation of 39 amino acids. This amino acid sequence was 85% homologous to *Homo sapiens* DcytB and 79% homologous to both rat (*Rattus norvegicus*) and mouse (*Mus musculus*).

Gene Expression of Fe Enzyme and Receptors in the Duodenum

Semiquantitative RT-PCR analysis revealed significantly ($P \leq 0.05$) increased mRNA expression in the small intestine duodenal segments of DMT1, ferroportin, and DcytB (an elevation of 13, 30, and 20%, respectively) in the low-Fe group compared with the high-Fe group (Figure 2).

Table 2. Body weights, feed and Fe intakes, blood hemoglobin concentrations, and hemoglobin maintenance efficiency (HME) in broiler chicken fed diets containing Fe-adequate maize-based diet (Fe+, 141 μg of Fe/g of diet) and Fe-deficient maize-based diet (Fe−, 51 μg of Fe/g of diet) from d 7 to 49[1]

Treatment	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49
BW (g)							
Fe+	143[a] ± 6.8	361[a] ± 12	776[a] ± 29	1,290[a] ± 39	1,867[a] ± 61	2,413[a] ± 127	2,828[a] ± 150
Fe−	130[a] ± 4.5	330[b] ± 11	707[b] ± 22	1,200[b] ± 43	1,736[b] ± 56	2,255[b] ± 95	2,647[b] ± 78
Fe intakes[2] (mg, cumulative weekly from d 0)							
Fe+	14.1[a] ± 1.5	33.8[a] ± 10.4	114.1[a] ± 9.8	247.1[a] ± 13.6	399.3[a] ± 18.6	642.9[a] ± 22.5	1,048.1[a] ± 25
Fe−	6.63[b] ± 0.7	14.2[b] ± 1.8	43.7[b] ± 7.9	90.7[b] ± 10.1	146.8[b] ± 16.0	235.8[b] ± 15.9	387.9[b] ± 13.6
Hemoglobin concentration (g/L)							
Fe+	145[a] ± 2.6	115[a] ± 2.9	110[a] ± 4.2	108[a] ± 4.6	105[a] ± 3.6	105[a] ± 5.1	107[a] ± 5.7
Fe−	145[a] ± 2.8	101[a] ± 4.9	92[b] ± 3.9	85[b] ± 4.1	80[b] ± 1.9	77[b] ± 3.5	72[b] ± 4.2
HME[3] (%)							
Fe+	—	17.4[a] ± 1.9	16.1[a] ± 1.1	13.7[a] ± 1.4	12.5[a] ± 1.4	12.1[a] ± 1.1	11.8[a] ± 1.5
Fe−	—	25.2[b] ± 2.9	28.9[b] ± 2.8	25.5[b] ± 2.2	22.9[b] ± 2.3	22.3[b] ± 2.1	21.9[b] ± 2.8

[a,b]Within a row, means without a common superscript are significantly different ($P < 0.05$).

[1]Values are means ± SEM, n = 10.

[2]Values are mean daily feed intakes for the 7 d proceeding the day designated in the column heading.

[3]Iron bioavailability was calculated as HME (Tan et al., 2008; Tako et al., 2009): $\text{HME} = \frac{\text{Hb Fe (final)} - \text{Hb Fe (initial)}}{\text{Total Fe intake, mg}} \times 100$, where Hb Fe = total body hemoglobin Fe. Hb Fe was calculated from hemoglobin concentrations and estimates of blood volume based on BW (a blood volume of 85 mL per kg of BW is assumed) (Sturkie, 2000): Hb Fe (mg) = BW (kg) × 0.085 L of blood/kg × Hb (g/L of blood) × 3.35 mg of Fe/g of hemoglobin.

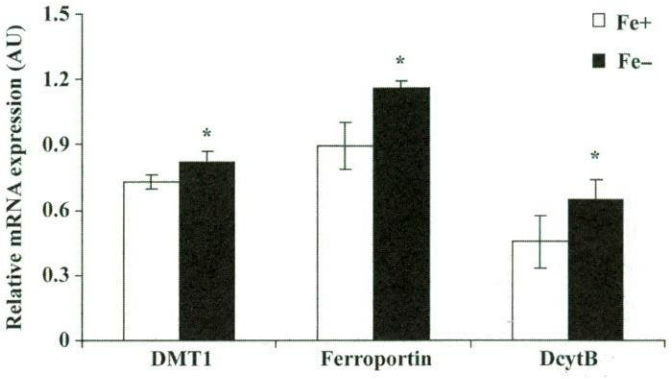

Figure 2. Chicken duodenal mRNA expression of divalent transporter 1 (DMT1), ferroportin, and duodenal cytochrome B (DcytB) in adequate-Fe (Fe+) and low-Fe (Fe−) birds. Changes in mRNA expression were measured by semiquantitative reverse transcription-PCR and expressed relative to expression of 18S rRNA in arbitrary units (AU). Values are means ± SEM, n = 6. An asterisk (*) indicates significantly different from the Fe+ treatment group ($P < 0.05$).

Caco-2 Cell Ferritin Protein Formation

Using an in vitro digestion-Caco-2 cell culture model, ferritin formation was 14.78 ± 0.9 ng/mg of protein in cells exposed to digests of the Fe-adequate diet compared with 5.18 ± 0.6 ng of ferritin/mg of protein in the Fe-deficient diet ($P < 0.05$), indicating that more Fe was taken up by the Caco-2 cells from the Fe-adequate diet.

Fe Absorption

Fe absorption rates of ^{58}Fe in the low-Fe birds were significantly higher than absorption rate in high-Fe birds. Average absorption rates were 22.11 ± 7.2% and 13.35 ± 4.6% for ^{58}Fe absorption in Fe-deficient and Fe-adequate birds, respectively (values are mean ± SEM, n = 3; Figure 3).

DISCUSSION

The main goal of this study was to evaluate the broiler as a model for assessment of Fe bioavailability via a 7-wk feeding trial and to introduce a unique duodenal loop technique for direct measurement of Fe absorption. Also, we isolated and sequenced a partial sequence of the chicken DcytB cDNA; DcytB is a brush border membrane enzyme that reduces Fe from the ferric (Fe^{+3}) to the ferrous (Fe^{+2}) state.

Iron absorption is regulated, in part, by intracellular Fe concentrations in enterocytes (Ludwiczek et al., 2004). Iron ions (Fe^{2+} and Fe^{3+}) reach the duodenal brush border membrane then are reduced by DcytB to Fe^{2+} (unless already in the Fe^{2+} form), which is then transported into the enterocyte via DMT1. Alternative mechanisms for Fe entry into enterocytes are possible and likely but have not been conclusively demonstrated (Kim et al., 2008). Within the cell, Fe is either stored as ferritin or trafficked to the basolateral membrane and exported into the circulation. Transport across the basolateral membrane is accomplished by the coordinated action of ferroportin, an Fe transporter, and hephaestin, which oxidizes Fe^{2+} to Fe^{3+}. Iron ions (Fe^{3+}) then bind to transferrin for distribution throughout the body via the plasma circulation (Collins et al., 2005).

Iron is a required element for the function of numerous enzymes and participates in oxygen transport and DNA synthesis. Therefore, Fe is a vital nutrient for the development of the fast-growing chick and the minimal

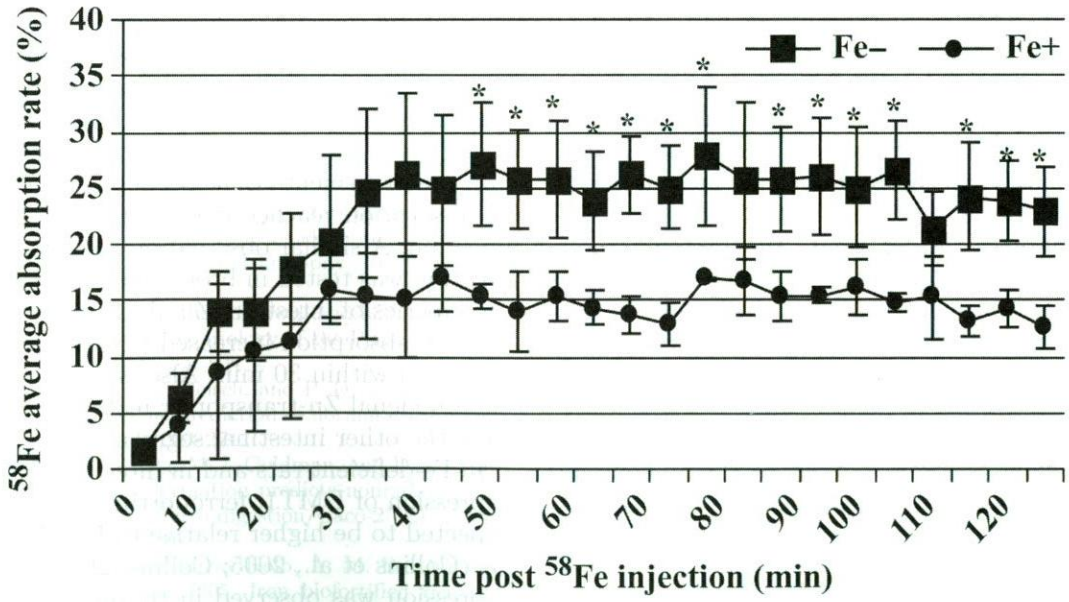

Figure 3. Duodenal loop Fe absorption rate. A comparison between adequate-Fe group (Fe+) that was given an Fe-adequate maize-based diet (141 μg of Fe/g of diet) and low-Fe group (Fe−) that was given an Fe-deficient maize-based diet (51 μg of Fe/g of diet). Blood samples were collected before stable isotope injection and then every 5 min and for 120 min post solution injection. Samples were analyzed by inductively coupled argon-plasma mass spectrometry for ^{58}Fe concentrations [absorption of ^{58}Fe (%); values are treatment means ± SEM, n = 3]. An asterisk (*) indicates significantly different from the Fe+ treatment group ($P < 0.05$).

dietary Fe requirement for broilers has been set at 80 mg of Fe/kg of diet (NRC, 1994). Dietary Fe deficiency was shown to affect the development of the chicken. For example, Fe deficiency affected the intestinal mucosa functionality by decreasing villus surface area (Sáiz et al., 1993). These observations supported our initial hypothesis that the chicken is sensitive to dietary Fe concentrations and therefore can be used as a model to test Fe bioavailability in foods.

In addition, the intestinal loops of broilers have been used for mineral absorption measurements. Previously, the broiler's duodenal in situ ligated technique was used to assess dietary glucose and Zn absorptions (Riesenfeld et al., 1980; Yu et al., 2008). However, relatively little information is available on Fe absorption in poultry and on specific transport in chicken intestines.

A poultry model for Fe bioavailability studies can have numerous applications, but in general, it can be used to identify foods, food combinations, and factors within diets that can help prevent Fe deficiency anemia. It may be especially useful in the strategy of biofortification. This approach uses plant breeding to select for traits that enhance the nutritional quality of crops by increasing Fe concentration or bioavailability, or both (Haas et al., 2005). Because the North American poultry diet is composed primarily of maize, it may be especially useful to develop maize and other crops for improved Fe nutrition.

In biofortification studies, the effect of a biofortified food is expected to be preventative; thus, depending on the duration of the study, marginally Fe-adequate or Fe-deficient animals are desired. Anemic animals are not desirable for biofortification studies because physiological adaptation may mask differences in bioavailable Fe between test samples. Also, the difference in deliverable Fe may not reverse the anemia or require a longer time to show a measurable benefit. Alternatively, Fe-adequate animals may take a long time to show depletion of Fe; thus, less effect would be shown during a study. Therefore, the initial Fe status should be tailored to accommodate possible changes in Fe status and thus maximize the potential for measurement of physiological effects.

Given the above considerations, the present study provides information regarding how to set the Fe status of the birds for future biofortification studies. In the present study, the chicks were given a typical starter diet (NRC, 1994) during the first week posthatch (create Fe stores). At d 7, chicks were moved to the tested diets (low Fe vs. high Fe). This dietary treatment yielded what we were hoping to show. That is, an experimental group with Fe stores that were rapidly depleted by an Fe-deficient diet and exhibiting the expected upregulation of Fe absorption and expression of the Fe transporter and proteins involved in Fe absorption. These results document the expected response of the broiler to Fe deficiency and provide information as to how Fe status can be manipulated in this model. Such information is necessary so that future studies can be done comparing foods that may have differences in Fe bioavailability.

In addition, the current study documents the changes in body Hb Fe in the birds as measures of Fe absorption and uses absorption of an isotopic tracer to demonstrate the adaptive response of the duodenum to Fe deficiency.

The mean Hb concentrations in both groups at the start of the feeding period were about 145 g/L, and concentrations were maintained at this level throughout the study in the group receiving a high-Fe diet but fell significantly in the low-Fe diet group. In a previous study aimed to test Fe requirements of chicks fed a semipurified diet based on casein and soy protein, hatchlings that were fed the basal diet (Fe concentration was 46.5 μg/g of diet) had a final Hb concentration of 61 versus 89 g/L in chicks given the same diet supplemented with 50 mg of Fe/kg of diet. In addition, BW gains were 60% higher in the Fe-supplemented group (Aoyagi and Baker, 1995). In the present study, final total body Hb Fe contents and the increase in total body Hb Fe over the 7-wk feeding period were significantly greater in the high-Fe group versus the low-Fe group (84.6 ± 10.2 mg vs. 54.3 ± 9.8 mg, respectively). Also, the ^{58}Fe absorption rates in the low-Fe birds were significantly higher than in the high-Fe birds. Previously, it was shown that the intestinal Fe absorption in 7-wk-old chicken and after 60 min of perfusion was 19% (Sáiz et al., 1993). In this study, we observed a similar Fe absorption rate in the duodenal loop.

We chose to measure Fe absorption in the duodenal loop because the duodenum of poultry, similar to humans, is the major intestinal site of Fe absorption (Sáiz et al., 1993; Aoyagi and Baker, 1995). The plots of Fe absorption percentage against time post intraluminal injection of the stable Fe isotope showed that there were asymptotic time-dependent increases of Fe absorption. These results are in agreement with similar findings of magnesium and other mineral elements absorption in mammals (Thomson et al., 1971). For example, the rate of pig intestinal Zn transport has decreased as from 30 min posttreatment (Blakeborough, 1987). In our study, Fe absorption reached equilibrium within 40 min after injection. A similar observation was made when Zn absorption was tested in broilers in a trial aimed to study the kinetics of intestinal Zn absorption. Results showed that Zn absorption increased linearly and then reached a plateau within 30 min. Also, similar to Fe transporters, duodenal Zn transporter mRNA levels were higher than the other intestinal segments (Yu et al., 2008).

In Fe-deficient rats and in intestinal cell cultures, the expression of DMT1, ferroportin, and DcytB mRNA is expected to be higher relative to Fe-adequate individuals (Collins et al., 2005; Collins, 2006). This pattern of expression was observed in the present study and was described in Fe-deficient rats and in vitro (Johnson et al., 2005). Previously, it was shown that the elevated gene expression for these transporters and enzymes is due to the dietary Fe deficiency conditions and increas-

es cellular Fe uptake and export into the circulation (Johnson et al., 2005). These observations indicate that the Fe uptake mechanisms in the broiler are responding as expected to dietary Fe.

So far, rats or piglets were used as models for Fe bioavailability studies due to similarities in gastrointestinal anatomy and physiology to humans. However, rodent-piglet long-term feeding trials are costly, hard to maintain, and limited by the number of animals. In contrast, chickens are cheaper and easier to maintain. Also, the chicken intestinal anatomy allows using the duodenal loop for in situ dietary Fe bioavailability tests. Moreover, the overall size of the adult broiler allows for repeated blood sampling at volumes suitable for measurement of trace minerals. Further studies on the loop model are needed to determine its sensitivity and usefulness in Fe bioavailability of foods.

Based on the present data, we conclude that the in vivo results confirmed the in vitro observations and that these findings suggest that the broiler model mimics the physiological effects of Fe deficiency as reported in other species. As such, the duodenal loop method shows promise as an intermediate, accessible, and relatively cheap screening tool for Fe availability and link between in vitro observations and human studies.

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